

The University of Chicago

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THE OXIDATION OF PYRUVIC ACID
IN PIGEON BREAST MUSCLE

A DISSERTATION SUBMITTED TO THE FACULTY OF
THE DIVISION OF THE BIOLOGICAL SCIENCES IN
CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
JUNE, 1945

By
LESTER RICE

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In 1938 Krebs and Eggleston (1) demonstrated an in vitro stimulation by insulin of the oxygen uptake of a suspension of minced pigeon breast muscle in phosphate buffer. The effect was especially pronounced when various oxidizable substances were added, in particular citric acid. Though the site of action of insulin in this experimental system was unknown, Krebs and Eggleston concluded that their evidence "suggests that insulin acts as a catalyst in the citric acid cycle". Several publications have confirmed and extended Krebs and Eggleston's observation. Shorr and Barker (2) showed that under the conditions employed by Krebs and Eggleston the effect could be shown in minced pigeon breast muscle but not in the minced muscles of the chicken, dog, rabbit, and cat. Using pigeon breast muscle, Stadie, Zapp and Lukens (3) found citrate ineffective in enhancing the effect of insulin on oxygen uptake although the hormone caused an increased respiration. Stare and Baumann (4) demonstrated that the addition of malonic acid to the tissue suspension at the start of the experiment abolished the insulin effect and that the hormone maintained the respiratory quotient of pigeon breast muscle at a value near one. They also observed larger insulin effects when muscle from depancreatized birds was used. Stadie (5) tested the action of insulin on the respiration of malonate-poisoned pigeon breast muscle to which varying amounts of fumarate were added. Although the vigorous metabolism obtained in this preparation is believed to take place mainly through the citric acid cycle, insulin did not affect the rate of oxygen uptake during the two hour long experiment.

It seemed possible to us that information in regard to the action of insulin in the experimental system used by Krebs and Eggleston (1) could be obtained by studying the respiration

of a suspension of minced pigeon breast muscle during the period when the insulin effect is present.

In experiments to this end, we have found, first, that the greater oxygen uptake of a suspension of pigeon breast muscle to which insulin had been added (as compared to a control maintained under the same conditions for a similar period of time) is accompanied by an increased ability to utilize pyruvic acid.

We have found, further, that this pyruvate utilization can be inhibited by malonate and restored by the simultaneous addition of fumaric acid and pyruvic acid or of pyruvic and oxaloacetic acids. While both reactions initiated by the addition of these pairs of acids occur at a greater rate in the insulin-supplemented tissue, the rates of citric acid and α -ketoglutaric acid oxidation are unaffected by the presence of the hormone.

A similar effect of insulin can be demonstrated to occur with pigeon liver. However, the hormone has no demonstrable in vitro effect on the condensation of acetoacetic acid and oxaloacetic acid which occurs in kidney tissue (6) (7).

EXPERIMENTAL

All organic acids were used as sodium salts which were prepared by neutralizing the solid acid or a concentrated solution of the acid with 1.0 M sodium bicarbonate and diluting to volume. Pyruvic, oxaloacetic and α -ketoglutaric acid solutions were prepared fresh just before use. Boiled muscle extract was prepared by adding an equal volume of water to minced pigeon breast muscle, heating for ten minutes in a boiling water bath and filtering.

The insulin used was an amorphous, highly purified preparation obtained from Eli Lilly and Company which assayed 22

units to the milligram. It was dissolved in M/10 phosphate, pH 7.4.

All manometric experiments were run at a temperature of 40° C. using the Warburg apparatus. The vessels were gassed with 100% oxygen.

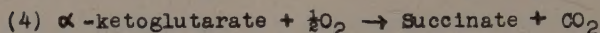
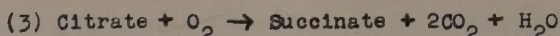
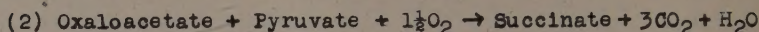
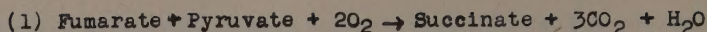
Pyruvic acid was determined by the carboxylase method (8). Acetoacetic acid alone was determined by the aniline acetate method of Edson (9); while the method of Van Slyke (10) was used if both acetoacetic and β -hydroxy-butyric acids were to be measured. Succinic plus α -ketoglutaric acid was extracted and analyzed for by the method of Krebs and Eggleston (8). The directions of Krebs, Smythe, and Evans (11) were followed in measuring fumaric acid. The method of Fucher, Sherman and Vickery (12) was used in analyzing for citrate.

Results

The Action of Insulin on Minced Pigeon Breast Muscle.--

The effect of insulin on pyruvic acid oxidation on pigeon breast muscle is demonstrated in Table 1. In the absence of insulin the quantity of oxygen consumed by the tissue exceeds that required for the total oxidation of the pyruvic acid disappearing; in the presence of insulin the complete oxidation of pyruvic acid to CO_2 and H_2O accounts for practically the whole of the oxygen uptake.

Since the oxidation of pyruvic acid by pigeon breast muscle proceeds by reactions 1-4 (8) (13), it was possible to ascertain which of these component reactions was influenced by the hormone by adding to the tissue suspensions sufficient malonate to block the oxidation of succinic acid and then to measure the rate at which reactions 1-4 occurred. The tissue suspensions were incubated at 40° C. in an atmosphere of oxygen until the



insulin effect appeared, malonate and the various substrates added and measurements of oxygen uptake and substrate removal carried out. The data in Table 2 show that while insulin has little effect on the slow rate of pyruvate removal by the malonate poisoned tissue, the rate at which pyruvate is removed when fumarate or oxaloacetate are also present is definitely accelerated in the insulin supplemented tissue.

The effect of insulin on pyruvic acid utilization consists therefore in maintaining the ability of the tissue suspension to carry out reactions 1 and 2. According to present views (13), these reactions involve a condensation of pyruvic acid and oxaloacetic acid to yield a tricarboxylic acid (aconitic acid). This reaction, rather than the subsequent oxidation of the tricarboxylic acid to α -ketoglutaric acid or succinic acid is involved in the insulin effect.

In tables 3, 4, and 5, the same conclusion is demonstrated to follow from experiments in which measurements of the various substrates used, as well as of oxygen consumption, were carried out.

The addition of boiled muscle extract not only increased the oxygen consumption of the tissue suspensions but was necessary for demonstrating the insulin effect. It is probable that the usual tissue suspension contains only sub-optimal amounts of some substances necessary in the pyruvic acid oxidizing system and that the addition of boiled muscle extract increases their concentrations to a point where the quantity of insulin present

becomes the major rate determining factor in the system.

The Action of Insulin on Minced Pigeon Liver.--A study of the effect of insulin on pigeon liver suspensions was made by one of us (E. A. E.) in 1940 at the suggestion, and in the laboratory of Dr. Hans A. Krebs. In these unreported experiments, insulin and substrates were added simultaneously at the beginning of the experimental period. While a stimulating effect of insulin was frequently observed under these conditions, it proved impossible at that time to define the conditions necessary for a demonstration of the phenomenon. However, the procedure described in the present report yields sufficiently consistent results to permit a demonstration that the increase in oxygen uptake caused by the addition of insulin to the medium is concerned with the utilization of pyruvic acid. The experimental procedure employed was essentially that used in the experiments of Table 1. Vessels with and without insulin were shaken for approximately 120 minutes at which time the insulin effect had become pronounced. Pyruvate was then tipped in. As with pigeon breast muscle, the insulin-containing vessel showed an increased oxygen uptake and a greater utilization of pyruvic acid as compared to the control (Table 6).

The Utilization of Acetoacetic and Pyruvic Acids in Rabbit Kidney.--Wieland and Rosenthal (7) have shown recently that an oxidative condensation of acetoacetic acid with oxaloacetic acid to yield citric acid occurs in kidney tissue. The similarity between this reaction and the initial step in the utilization of pyruvic acid in muscle tissue led Wieland and Rosenthal (7) to suggest that insulin might be concerned in the oxidation of acetoacetic acid by kidney.

Wieland and Rosenthal measured only citric acid formation

after the addition of various substrates to beef and rabbit kidney suspensions. Barium chloride was used to inhibit citric acid removal in beef kidney suspensions but no inhibitor was used with rabbit kidney. Nearly all the reported experiments dealt with beef kidney suspensions.

In the present work rabbit kidney suspensions were used exclusively. Not only citrate formation, but acetoacetic acid removal and oxygen uptake were measured. Fumaric acid rather than oxaloacetic acid was used as the catalytic dicarboxylic acid since it did not interfere with the analysis of acetoacetic acid by the aniline acetate method (9) (this involves measuring the CO_2 split off from α -keto acids). In our experience concentrations of barium chloride similar to those used by Wieland and Rosenthal (7) in beef kidney suspensions did not affect appreciably the rate of citric acid disappearance in minced rabbit kidney. The barium salt, however, markedly increased the rate of acetoacetic acid utilization and oxygen uptake (table 7). When higher concentrations of barium chloride were tested, the metabolic activity of the kidney suspension was strongly depressed.

It was possible to measure citric acid formation in the kidney suspensions by using trans-aconitic acid.¹ A 0.025 M concentration of trans-aconitic acid caused virtually complete inhibition of citric acid breakdown. Trans-aconitate did not interfere with the citric acid analysis. Table 8 summarizes an experiment on rabbit kidney suspension by which the utilization of acetoacetic acid in this tissue was investigated. Acetoacetic acid utilization, citric acid formation and oxygen uptake were

¹Dr. James W. Moulner, in this laboratory had, while working with extracts of acetone-dried pigeon liver powder, obtained evidence that trans-aconitic acid could inhibit the oxidation of citric acid. Samples of pure trans-aconitic acid were kindly furnished by him.

measured. The results obtained agree with the conclusion of Wieland and Rosenthal (7) that acetoacetic acid is oxidized to citric acid. Insulin did not increase the rate of oxygen uptake or acetoacetic acid removal. Barium chloride increased the rate of acetoacetic acid utilization.

Wieland and Rosenthal (7) found that, in beef kidney, oxaloacetic plus pyruvic acid formed, in a given time, only half the amount of citric acid obtained when oxaloacetic plus acetoacetic acid were added. The rates of pyruvic and acetoacetic acid utilization were not measured. On the basis of this evidence they suggested that pyruvic acid oxidation in this and other tissues involved its preliminary conversion to acetoacetic acid. This would then be oxidized to citric acid.

We have repeated the above experiment, measuring pyruvic and acetoacetic acid utilization, citric acid formation and oxygen uptake. The results indicate that in rabbit kidney suspensions pyruvic acid is removed more rapidly than acetoacetic acid. In contrast to the rate of acetoacetic acid utilization the rate of pyruvic acid utilization is not decreased by trans-aconitic acid or materially increased by fumaric acid. Pyruvic acid itself yields only small amounts of citric acid (Table 9). Consequently, under the conditions existing in this tissue preparation, the utilization of pyruvic acid seems to occur in a somewhat different manner than that of acetoacetic acid.

Minced pigeon breast muscle was found to utilize only small amounts of acetoacetic acid. This was mainly converted to β -hydroxy-butyric acid. Insulin did not increase acetoacetic acid utilization in this tissue.

TABLE 1

THE EFFECT OF INSULIN ON AEROBIC PYRUVATE REMOVAL

Two flasks contained 2.5 gm. of minced pigeon breast muscle in 22.5 ml. of calcium-free phosphate saline (pH 7.4) plus 5.0 ml. boiled muscle extract. One flask received 1.5 ml. phosphate buffer; the other, 1.5 mg. zinc-free insulin in a 1.5 ml. phosphate buffer. Both vessels were gassed with 100% O₂. 4.0 ml. samples from each flask were placed in Warburg vessels, gassed with 100% O₂ and shaken at 40° C. until these pilot vessels showed the beginning of the insulin effect (ca. 80 minutes). The reserve flasks which had been shaken at 40° C. during this time were removed from the water bath and 4.0 ml. of the enzyme suspensions plus other additions were added to Warburg vessels as indicated in the table. The vessels were gassed with 100% O₂, equilibrated at 40° C. for 10 minutes and substrates tipped in from the side arm. 20% KOH was placed in the center cup. Total volume of liquid: 417 ml. Experimental period: 25 minutes. Pyruvic acid was measured by the carboxylase method.

Experiment	1		2		3	
Insulin added (units).....	-	5	-	5	-	5
Pyruvate added (μl).....	431	431	373	373	393	393
Pyruvate utilized (μl)....	92	234	69	191	224	300
Oxygen uptake (μl).....	390	475	441	475	634	944

TABLE 2

EFFECT OF INSULIN ON THE O_2 UPTAKE AND PYRUVATE REMOVAL
IN MALONATE POISONED SYSTEMS

All manipulations are the same as those recorded in Table 1. Malonate was added directly to all vessels to give a final concentration of 0.019 M. The vessels were run for 70 minutes. The data in this table are from the same tissue suspensions used in Experiment 2, Table 1. Vessels 7 and 8 contained 336 μ l of $MgSO_4$.

	Vessel									
	1	2	3	4	5	6	7	8	9	10
Insulin added (units).....	-	5	-	5	-	5	-	5	-	5
Fumarate added (μ l).....	-	-	224	224	-	-	-	-	-	-
Oxaloacetate added (μ l).....	-	-	-	-	224	224	-	-	-	-
Citrate added (μ l).....	-	-	-	-	-	-	448	448	-	-
α -ketoglutarate added (μ l).....	-	-	-	-	-	-	-	-	448	448
Pyruvate added (μ l).....	373	373	373	373	373	373	-	-	-	-
Pyruvate re-covered (μ l).....	275	246	235	167	360	311	-	-	-	-
Pyruvate utilized* (μ l)....	98	127	138	206			-	-	-	-
Oxygen uptake (μ l).....	159	194	272	452	222	282	183	192	164	154

*Calculation of pyruvate utilization in the presence of oxaloacetate is impossible since oxaloacetate yields 2 mols of CO_2 in the carboxylase method and is also de-carboxylated to an unknown degree when added to tissues. These data, however, indicate an increased pyruvate utilization in the presence of insulin and oxaloacetate.

TABLE 3

THE EFFECT OF INSULIN ON THE REACTION:
 $\text{FUMARATE} + \text{PYRUVATE} + 2\text{O}_2 \rightarrow \text{SUCCINATE} + 3\text{CO}_2 + \text{H}_2\text{O}$

The enzyme was prepared by mixing 5.0 gm. of fresh minced pigeon breast muscle plus 45.0 ml. of ice-cold calcium-free phosphate saline (pH 7.4) plus 10.0 ml. of boiled muscle extract. 4.0 ml. of enzyme were added to each Warburg vessel. Insulin was added to the reaction chamber at the beginning of the experiment. The vessels were gassed with 100% O_2 , equilibrated at 40°C . and run at this temperature until an insulin effect on the oxygen uptake appeared (ca. 70 minutes). They were then removed from the bath, malonate added directly to the reaction chamber while other substances were added to the side arm. The vessels were regassed, reequilibrated, tipped and run to the conclusion of the experiment at which time the vessel contents were analyzed for the substituents listed below. All vessels contained malonate which was present in a concentration of 0.019 M. 20% KOH was placed in the center cup. Total volume of liquid present in each vessel was 5.2 ml. Experiment 1 lasted 52 minutes while the duration of Experiment 2 was 89 minutes.

	Experiment 1		Experiment 2	
	I*	NI*	I*	NI*
Pyruvate added (μl).....	325	325	320	320
Fumarate added (μl).....	336	336	330	336
Pyruvate utilized (μl).....	233	115	228	94
Succinate formed**(μl).....	227	91	236	114
Fumarate utilized (μl).....	248	112	189	82
Citrate formed (μl).....	13	19	0	0
Oxygen uptake (μl).....	590	340	566	200

*The columns labeled I denote the vessels containing insulin (1.2 units per ml.). Those labeled NI denote vessels containing no insulin. This notation will be used in the following tables.

**In this and succeeding tables, the term "succinate formed" actually stands for the succinate plus α -ketoglutarate present. Krebs (8) has shown that this value represents about 90% true succinate.

TABLE 4

THE EFFECT OF INSULIN ON THE REACTION:
 $\text{OXALOACETATE} + \text{PYRUVATE} + \frac{1}{2}\text{O}_2 \rightarrow \text{SUCCINATE} + 3\text{CO}_2 + \text{H}_2\text{O}$

In this experiment reaction (b) (see page 4) was compared with reaction (a). All manipulations and conditions were identical with those observed in Table 3. The experimental period was 48 minutes.

	Oxaloacetate		Fumarate	
	I	NI	I	NI
Pyruvate added (μl).....	385	385	385	385
Fumarate added (μl).....	-	-	336	336
Oxaloacetate added (μl)....	670	670	-	-
Pyruvate recovered (μl)†...	306	491	92	192
Pyruvate utilized (μl).....			293	193
Succinate formed (μl).....	291	158	254	148
Citrate formed (μl).....	13	14	10	11
Oxygen uptake (μl).....	556	348	603	370

*See comments on pyruvate measurement made in Table 2.

TABLE 5

THE EFFECT OF INSULIN ON THE REACTION:
 $\text{CITRATE} + \text{O}_2 \rightarrow \text{SUCCINATE} + 2\text{CO}_2 + \text{H}_2\text{O}$

All manipulations and conditions employed were identical with those described for the experiments in Table 3. Although not shown in the table, a fumarate-pyruvate system was run simultaneously to confirm the viability of the tissue and the presence of an insulin effect. Experiment 1 ran 66 minutes; Experiment 2, 85 minutes. All vessels contained 450 μl of Mg^{++} ion.

	Experiment 1		Experiment 2	
	I	NI	I	NI
Citrate added (μl).....	336	336	336	336
Citrate utilized (μl).....	70	70	76	73
Succinate formed (μl).....	75	29	62	49
Oxygen uptake (μl).....	221	194	183	179

TABLE 6

THE EFFECT OF INSULIN UPON AEROBIC UTILIZATION
IN MINCED PIGEON LIVER

A pigeon was decapitated, its liver rapidly removed and chilled on ice. The liver was put through a Fischer hand mincer, dispersed in ice-cold M/10 phosphate buffer (pH 7.4) and sufficient buffer added to make the proportions one part liver to three parts buffer. The suspension was used directly.

3.0 ml. samples of the enzyme were placed in Warburg vessels. Insulin was added to those vessels being supplemented with the hormone. Pyruvate was placed in the side arm. The vessels were gassed with 100% oxygen, equilibrated at 40° C. for 10 minutes and run until an insulin effect appeared (ca. 120 minutes). The pyruvate was tipped in from the side arm and the experiment run to its conclusion. 20% KOH was placed in the center cup. Total volume of liquid in the vessel was 3.6 ml. After tipping, Experiment 1 ran 70 minutes while Experiment 2 ran for 105 minutes.

	Experiment 1		Experiment 2	
	NI	I*	NI	I*
Pyruvate added (μ l).....	652	652	645	645
Pyruvate utilized (μ l).....	425	645	345	468
Oxygen uptake (μ l).....	966	1657	804	1355

*The insulin concentration was 3 units per ml.

TABLE 7

THE EFFECT OF BARIUM CHLORIDE AND INSULIN ON
THE OXIDATION OF ACETOACETATE

The kidneys were rapidly removed from a rabbit and chilled. They were put through a hand mincer and the mince obtained mixed with $2\frac{1}{2}$ ml. of ice-cold Krebs calcium-free phosphate buffer (pH 7.4) for every gram of mince. The mixture was used directly. 3.0 ml. of the enzyme suspension were added to the main chamber of a Warburg vessel together with the barium chloride and insulin. The other additions were put into the side arm. The vessels were gassed with 100% oxygen, equilibrated at 40° C. for 10 minutes, then tipped and run to the completion of the experiment. Analyses were run on the vessel contents. 20% KOH was placed in the center cup. Total volume of liquid present was 3.85 ml.

Vessel.....	1	2	3	4	5	6
Acetoacetate added (μ l).....	470	470	470	470	470	470
Fumarate added (μ l).....	-	900	900	900	900	900
BaCl ₂ added (μ l).....	-	-	900	900	900	900
Insulin added (μ l).....	-	-	-	5	-	5
Acetoacetate utilized (μ l)...	97	156	464	446	461	443
Citrate found (μ l).....	0	0	0	23	61	45
Oxygen uptake (μ l).....	1376	2497	2764	2388	1682	1574
Time run (minutes).....	100	100	100	100	46	46

TABLE 8

THE CONVERSION OF ACETOACETATE TO CITRATE

The procedure and conditions used were identical with those in Table 7 except that 30 minutes elapsed before the contents of the side arms were tipped in. Trans-aconitate was placed in the side arm. The duration of the experiment was 75 minutes after tipping. The vessel contents totaled 4.05 ml.

Vessel.....	1	2	3	4	5	6
Acetoacetate added (μ l)...	502	502	502	502	502	502
Fumarate added (μ l).....	900	900	900	900	900	900
BaCl ₂ added (μ l).....	448	-	448	448	-	-
Trans-aconitate added (μ l)	-	-	2010	2010	2010	2010
Insulin added (units).....	-	-	-	5	-	5
Acetoacetate used (μ l)....	456	158	272	219	116	98
Citrate found (μ l).....	71	15	368	276	180	173
Oxygen uptake (μ l).....	2768	2163	1635	1497	1483	1412

TABLE 9

A COMPARISON OF PYRUVATE AND ACETOACETATE
METABOLISM IN MINCED RABBIT KIDNEY

The procedure and conditions were as described previously in Tables 7 and 8. The incubation period was 20 minutes. Time of the experiment was 59 minutes and the vessel contents totaled 3.8 ml.

Vessel.....	1	2	3	4	5	6
Acetoacetate added (μ l)...	433	433	-	-	-	-
Pyruvate added (μ l).....	-	-	500	500	-	500
Fumarate added (μ l).....	900	900	900	900	900	-
BaCl ₂ added (μ l).....	-	448	-	448	-	-
Trans-aconitate added (μ l)	2020	2020	2020	2020	2020	2020
Acetoacetate used (μ l)....	155	315	-	-	-	-
Pyruvate used (μ l).....	-	-	398	461	-	409
Citrate found (μ l).....	159	367	168	266	124	18
Oxygen uptake (μ l).....	2305	2187	2358	2007	2132	1091

DISCUSSION

The hyperglycemia of a diabetic or depancreatized animal can originate from a decreased utilization of glucose by the animal, from an excessive production of glucose in its tissues, or from a combination of both conditions. The so called "underutilization theory" (which maintained that no oxidation of carbohydrate occurred in the peripheral tissues of the diabetic animal) was based mainly upon measurements of the respiratory quotient and the demonstration that the diabetic animal quantitatively excreted administered glucose (14). However, utilization of carbohydrate by the peripheral tissues of the diabetic animal has been conclusively demonstrated and there now seems no question that the diabetic animal can metabolize appreciable amounts of carbohydrate (15)(16)(17)(18)(19). The ability of the liver to convert fat to carbohydrate has also been confirmed (20)(21)(22). Consequently, an analysis of carbohydrate balance studies makes it likely that overproduction of glucose is a major factor in the hyperglycemia of the diabetic animal (23). However, the relative importance of overproduction and under-utilization in diabetic hyperglycemia has not been satisfactorily settled. Experiments with eviscerated dogs and rabbits have shown that the glucose utilization of the diabetic animal at its elevated blood sugar level is as great as that of the normal animal at a normal blood sugar level (17)(18). However, Greeley and Drury (18) have pointed out that the carbohydrate metabolism of these eviscerated animals is considerably lower quantitatively than that of active normal or depancreatized animals. It is possible, as several investigators have suggested

(16) (18), that appreciable differences in the rate of glucose utilization may exist between active normal and diabetic animals, even though the carbohydrate metabolism of these animals may be quantitatively similar at a basal, resting condition.

Therefore, the hyperglycemia of the diabetic animal may result not only from an excessive production of glucose from fat and protein in the liver but also from a simultaneous depression of carbohydrate utilization in the peripheral tissues. The effect which insulin exerts on the utilization of carbohydrate in various tissues makes this assumption more probable.

The present work has demonstrated that insulin causes an increased utilization of pyruvic acid through the tricarboxylic acid cycle in minced pigeon breast muscle. Although this is the first demonstration of an insulin effect on pyruvic acid utilization, a number of investigators have shown that the hormone influences other aspects of peripheral carbohydrate metabolism.

Soskin and Levine (17) have measured the rate of carbohydrate utilization in normal eviscerated and diabetic eviscerated animals at various blood sugar levels. The results indicated that, although diabetic dogs could utilize considerable carbohydrate, at a given blood sugar level the normal animal disposed of more glucose than the diabetic. It was only when high levels of glucose were maintained in the blood that normal and diabetic dogs showed the same rate of glucose utilization.

An effect of insulin on the carbohydrate utilization of isolated tissues has been shown by several investigators. Shorr (24) has found that diabetic tissues show lower respiratory quotients than do tissues from normal animals. He concluded that the effect of insulin resides in the oxidative rather than the glycolytic phase of muscle carbohydrate metabolism. Stadie (5) has

obtained both stimulation of respiration and elevation of the respiratory quotient in insulin-supplemented rat muscle extract. Cruickshank and Startup (25) have shown that insulin raises the respiratory quotient of the diabetic heart from 0.70 to 1.0 but does not increase its oxygen uptake. Glucose utilization in the working heart-lung preparation from diabetic animals is considerably lower than in preparations from normal animals. Addition of insulin to the circulating fluid raises the glucose utilization to normal levels (16)(26)(27). Isolated rat muscle suspended in a glucose-containing medium shows an increased rate of glycogen deposition in the presence of insulin (28)(29).

The work of Krebs and others showing an effect by insulin on the respiration of minced pigeon breast muscle has been discussed in the experimental section (1)(2)(3)(4). Bergenstal (30), in experiments with rat liver slices, has found that proper adjustment of the sodium and potassium concentrations of the medium permitted the demonstration of a stimulation of respiration by insulin. The magnitude of this stimulation could be increased by the addition of various oxidizable substrates to the medium. Best results were obtained with pyruvic and succinic acids but hexose-diphosphate, hexose-monophosphate, citric acid and oxaloacetic acid also gave good responses. A number of other compounds were tested and found to be negative.

Since recent investigations have demonstrated the important role which phosphate compounds play in carbohydrate metabolism (31)(32), the discovery that insulin influences the metabolism of phosphate takes on considerable significance. Sacks (33) has shown that the administration of insulin to cats injected with radioactive inorganic phosphate caused an increased uptake of radioactivity by the adenosine triphosphate and phosphocreatine

fractions of cat muscle; glucose plus insulin gave similar results plus a rise in the turnover of hexose-monophosphate. Nelson and coworkers (34) have studied the effects of insulin injection on the phosphate fractions of rat liver. They found an increase in the inorganic and easily-hydrolyzable acid-soluble phosphate fractions. Kaplan and Greenberg (35)(36)(37) have published a series of papers dealing with the effect of insulin on the phosphate fractions of rat liver. Injection of the hormone into the intact rat caused a rise in the amount of phosphorylated glucose intermediates, in the total acid soluble phosphate, and, significantly, in the amount of adenosine triphosphate in the animal's liver. The rate of adenosine triphosphate turnover as measured by p^{32} was markedly increased by insulin. Similar results were obtained after the injection of glucose alone. Even greater increases in the previously noted phosphate fractions were found after the injection of glucose plus insulin; glucose plus insulin also increased the turnover rates over those obtained with insulin or glucose alone. Kaplan and Greenberg concluded that insulin acts by stimulating carbohydrate utilization in a manner which increases the number of high energy phosphate bonds formed. This conclusion was strengthened by their finding that malonic acid inhibited formation of the extra adenosine triphosphate which normally occurs after the injection of insulin.

The pigeon has been found peculiarly suitable for demonstrating an in vitro effect by insulin on carbohydrate metabolism. The work reported in this paper as well as that of other investigators has been done on this animal (1) (2) (3) (4). Objections have been raised concerning the suitability of the pigeon as an experimental animal for studies on the mechanism of insulin action (23), and are based on the fact that the depancreatized pigeon

shows none of the symptoms found in depancreatized dogs and cats or in human diabetics (4)(38)(39). However, this is not an unusual response. As early as 1893, Minkowski (38) showed that herbivorous animals, in contrast to carnivores, survived extirpation of the pancreas very well. An ability to undergo pancreatectomy without becoming acutely ill has been shown in the following herbivorous animals; pigeons, ducks, and chickens (4) (38) (39) (40) (41) (42); pigs and goats (43)(44); rabbits (45); and rats (46) (47). Monkeys generally show mild symptoms after pancreatectomy (48)(49), though Mirsky and coworkers (50) have reported that more pronounced symptoms occur when the depancreatized animals are fasted.

Though insulin has been shown to profoundly influence the metabolism of the cat and dog, the hormone is not essential for their survival. The depancreatized-hypophysectomized or -adrenalectomized animal survives for long periods of time and shows a fairly normal carbohydrate metabolism without the administration of insulin (51)(52)(53)(54)(55). Recent work has indicated that completely depancreatized humans have a lower daily insulin requirement than severe human diabetics (56).

It can be concluded that the response of the pigeon to pancreatectomy is not abnormal but is characteristic of a herbivorous animal. The pigeon possesses a functional pancreas. Its pancreatic islands contain beta cells with secretory granules. The activity of these cells appears to be increased on histological study after partial pancreatectomy, forced feeding and the injection of pituitary hormone; procedures which stimulate insulin secretion (57). It has also been shown that the pigeon's endocrine glands function in the same manner as the endocrine systems of other animals (58). Therefore, although the bird is capable of

rapidly initiating a type of metabolism which does not require insulin once the pancreas is removed, insulin seems to play a role in its metabolism when the pancreas is present.

Although our results show that insulin causes a more rapid disappearance of pyruvic acid in pigeon breast muscle, the rate of pyruvic acid utilization in the pancreatectomized pigeon may not be as seriously impaired as these data suggest. Since the depancreatized bird appears capable of utilizing carbohydrate, a qualitative transformation in its mode of pyruvic acid utilization may have occurred. This might involve the oxidative utilization of pyruvic acid through mechanisms other than the tricarboxylic acid cycle or through a modification of the cycle which does not involve insulin. Kaplan and Greenberg (37) have shown that insulin affects the qualitative manner in which lactic acid is utilized by the rat liver *in vivo*. Little work has been done on the intermediary metabolism of depancreatized animals (24).

The metabolism of pyruvic acid through the tricarboxylic acid cycle not only results in a rapid and complete oxidation of this substrate but stimulates the formation of large amounts of energy-rich phosphate (31)(32). The discovery that insulin affects the oxidative utilization of pyruvic acid therefore permits a more precise explanation of the results obtained by Kaplan and Greenberg (36)(37) and by Bergenstal (30) which were discussed above. The stimulation of the reactions of the tricarboxylic acid cycle by insulin which their results indicated is probably due to an increased utilization of pyruvic acid through an oxidative condensation effected by the hormone. This mechanism of insulin action also allows certain conjectures to be made as to how insulin mediates its more general effects. Since the entrance of glucose into the metabolic cycles of muscle and other tissues seems to depend upon its initial phosphorylation, the catabolism

of pyruvic acid through the tricarboxylic acid cycle under the influence of insulin could stimulate greater utilization of blood sugar (5). These conjectures are consistent with what is actually observed. Insulin has been shown to cause an increased phosphate metabolism. The increased rate of glycogen deposition caused by insulin probably involves an increased rate of phosphorylation of blood glucose. A more rapid phosphorylation of blood glucose might also explain the greater rate of carbohydrate utilization of the normal over the diabetic animal at the same blood sugar level, while the complete and rapid removal of substrates which are oxidatively utilized, as is pyruvic acid, would partly account for the higher respiratory quotient of the normal animal over the diabetic. It is also possible that a cellular mechanism similar to that found in muscle is involved in the effect of insulin on the gluconeogenic, glycogenic, and glycogenolytic reactions of the liver.

SUMMARY

1. Insulin has been shown to increase the utilization of pyruvic acid in suspensions of minced pigeon breast muscle and minced pigeon liver.

2. Insulin functions in pigeon breast muscle by maintaining the functional integrity of the enzyme systems involved in the reactions of fumaric and pyruvic acids or of oxaloacetic and pyruvic acids.

3. The oxidative condensation of acetoacetic acid with fumaric acid to give citric acid has been confirmed in minced rabbit kidney.

4. Pyruvic acid is not utilized by the same mechanism as acetoacetic acid in rabbit kidney. Insulin did not influence either pyruvic acid or acetoacetic acid utilization in this tissue.

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